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Research article

Comparison of the sensitivity and cross-reactivity between heat-killed and formalin-killed cells during the development of indirect competitive ELISA for the detection of *Salmonella* Typhimurium

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ABSTRACT

Newer discovery of Salmonella strains warranted the development of newer ELISA system. The aim of this works is to develop a sensitive and simple indirect ELISA for the detection of *Salmonella* Typhimurium using formalin- and heat-killed cells as immunogens. The parameters of the resultant calibration curve for the formalin-killed cells (FKC) were minimum absorbance of 0.101± 0.011, maximum absorbance of 2.804± 0.020, slope parameter of 1.197, while c, the concentration that results in 50% response was 4.22 x 10^6 CFU/ml. The LOD and LOQ were 19,300 and 52,700 CFU/mL, respectively. The parameters of the typical calibration curve for heat-killed cells (HKC) were minimum absorbance of 0.115± 0.013, maximum absorbance of 2.810± 0.021, slope parameter was 1.441, while c, the concentration that results in 50% response was 2.15 x 10^6 CFU/ml. The LOD and LOQ were 47,100 and 123,000 CFU/mL, respectively. Antibody raised to FKC was twice the sensitivity and approximately ten times the linear range compared to antibody raised to HKC. Antibody produced from formalin-killed cells exhibited lower cross reactivity than heat-killed cells. The results of this work could open new avenues on optimization of antigen preparation for maximal sensitivity of the developed polyclonal antibody.

INTRODUCTION

Salmonella is a major foodborne pathogen globally. Its isolation has been reported from poultry products, poultry, raw meat, vegetables, milk and milk products [1,2]. Reheating of foods, poor cooking, and poor food handling of foods are the main contributor to Salmonella outbreaks. Diseases caused by Salmonella are generally called salmonellosis and include typhoid fever- which is the most lethal [3]. *Salmonella* Typhimurium (*Salmonella enterica* subsp. *enterica*, serovar Typhimurium) is one of Salmonellae paratyphoid most commonly associated with poultry. Thus, a detection assay for this bacterium is highly sought after. Conventional procedure for the detection of Salmonella is by the culture method. However, cultural isolation and identification requires three to five days for completion and includes tedious pre-enrichment, selective enrichment, selective agar plating, biochemical screening and serological identification [3]. Therefore, an immunological method has become widely used it is rapid, specific, highly sensitive and can detect a small amount of Salmonella in a sample [4]. Some methods may take only 2-3 hours for analysis, such as the ELISA, immuno-magnetic separation and the immuno-precipitation methods. However, the dot-ELISA method is a method of choice because it is easy to use, economical and highly sensitive [5]. In addition, it does not require special equipment and uses lower amounts of reagents.

New discovery of newer pathogenic Salmonella strains occur frequently including nonculturable Salmonella spp. [6] and this warranted the development of newer ELISA system based on polyclonal antibodies raised against new strains. Two methods of antigen preparation- heat- and formalin-killed cells will be developed and evaluated.

MATERIAL AND METHODS

Selective media and reference strains

Salmonella Typhimurium ATCC 53648, *Salmonella* Enteritidis ATCC 53000, *Salmonella* Pullorum 10398, *Salmonella* Gallinarum ATCC 1984 and *Campylobacter jejuni* ATCC 33291 were supplied by American Type Culture Collection (Manassas, VA, USA), *Escherichia coli* O157:H7 EDL933 was procured from the Faculty of Food Science and Biotechnology, Universiti Putra Malaysia. *Listeria monocytogenes* IMRL54108B was obtained from the Institute for Medical Research, Malaysia. Different microbiological media such as skim milk, peptone and nutrient broth (NB) were purchased from Oxoid, Hampshire United Kingdom.

Experimental animals

Accepted international standard on the care and use of experimental animals was used in the handling and preparation of rabbits for raising antibody [7]. Two months New Zealand White rabbits weighing between 2.0 to 2.5 kg were used in this experiment. Standard pellet diet was used and the animal house were set up under the following conditions: humidity (55%), temperature of 27 ^{o}C, and cycles of light/ dark of 12 h/ 12 h.

Antigen preparation

The modified method of Siragusa and Johnson [1] was employed in the preparation of formalin-killed and heat-killed cells as immunogens during the production of polyclonal antibody. *S.* Typhimurium was cultured in nutrient broth for 16 h at 37 ^{o}C on an orbital shaker at 150 rpm. Cells were enumerated using the colony count method. About 1 x 10^{9} CFU/ml cells were formalin-killed (0.1%) for 24 hours at room temperature. The bacterial cells were harvested by centrifugation at 5,000 x g at 10 ^{o}C for 10 min. The pellet was washed several times with sterile phosphate buffered saline (PBS) (0.01 M) and resuspended in the same media and stored at 4 ^{o}C.

Immunization

About 0.5 ml of Freund's complete adjuvant was mixed with 0.5 ml of formalin- and heat-killed cells. One milliliter of this emulsion was injected at the marginal ear of the New Zealand White rabbits. Injections were repeated for three times in three weeks but Freund's incomplete adjuvant was used to replace the complete adjuvant. One month later a booster injection was given and this was repeated at monthly intervals. Two weeks after each boost the rabbits were bled for antibody titer determinations. Serum in the blood samples were separated through centrifugation at 10,000 x g for 30 min at 4 ^{o}C [8].

Preparation of IgG

Purification of IgG was carried out on Protein A Sepharose fast Flow column (GE Healthcare). Initially the ionic strength and the pH of the serum were adjusted by dialyzing overnight at 4 ^{o}C in 0.02 M sodium phosphate, pH 7.0. Equilibration of the column was carried out by passing 5 column volumes of binding buffer (0.1 M phosphate buffer, pH 7.0) at 1 ml/min. Sample was loaded and then washed with several column volumes of buffer until the eluant showed no protein signal measured at 280 nm. Elution of IgG was carried out by applying elution buffer (0.1 M citrate, pH 3.0) at the flow rate of 1 ml/min. To ensure that the eluant reach neutral pH quickly, each collection tubes were filled with 0.5 ml of 1 M Tris-Cl pH 8.0. Fractions with the highest amount of protein measured at 280 nm were pooled and collected. Homogeneity was checked using SDS-PAGE.

Indirect ELISA

The indirect ELISA method was adopted for this assay with slight modifications [9]. About 100 µl of formalin-killed *S.* Typhimurium cells were coated onto a 96-well microtiter plate and then incubated at 37 ^{o}C for 2 hr. Then the plate was washed three times with 0.01 M PBS pH 7.0 containing 0.05% Tween 20 (PBST). Nonspecific binding was blocked using 200 µl of about 1% bovine serum albumin (BSA) in PBS for 2 hr at 37 ^{o}C. After washing, appropriate dilutions of primary antibody (rabbit anti-*S.* Typhimurium IgG) in PBS containing 0.05% Tween 20 and 1% BSA ranging from 1:1000 to 1:64000 were added and incubated for 2 hr at 37 ^{o}C. Following washing, goat anti-rabbit IgG antibody conjugated with the enzyme alkaline phosphatase initially diluted in PBS containing 1% BSA and 0.05% Tween 20 (dilution 1: 10000) was added. This mixture was incubated for 2 hr at 37 ^{o}C. The substrate p-nitrophenyl phosphate (1 mg/ml) was initially prepared in 10% (w/v) diethanolamine pH 9.8. The substrate was added and incubated at room temperature for 45 minutes. In order to stop the reaction, 50 µl KOH was added to each well. The p-nitrophenyl phosphate was measured at 405 nm using an ELISA reader (Thermo Scientific Multiscan FC, USA). Each experiment was performed in triplicates.

Optimization of parameters for calibration curve

Various parameters such as concentrations of primary and secondary antibodies, coating antigen, and incubation time were

optimized in order to construct a calibration curve of ELISA for the determination of *S*. Typhimurium.

Specificity test

The strains used for the specificity test were *Campylobacter jejuni* ATCC 33291, *Escherichia coli* O157:H7 EDL933 and *Listeria monocytogenes* IMRL54108B.

Limits Of Detection And Quantification

The limits of detection (LOD) for the assay can be modeled using a four parameter logistic equation [10] as follow;

$$y = \frac{a-d}{1+\left(\frac{x}{c}\right)^{b}} + d$$

Where y, x, a, d, c, and b are absorbance, dose or dilution, maximum response, minimum response, concentration that cause 50% response and slope-like parameter, respectively. The limit of detection (LOD) was calculated as the average value of absorbance at blank concentration of bacteria at 3 standard deviations (SD). Limit of quantification (LOQ) was calculated as the limit detection with 10 SD. LOD, LOQ and regression analysis were calculated using four-parameter logistics available from PRISM non-linear regression analysis software from www.graphpad.com.

RESULTS

Antibody production

IgG harvested from rabbit serum and determined for antibody titer shows that the antibody production was successful compared to pre-immune (Fig.1). This suggests that the immunization and purification method used were successful. The highest antibody production was second bleed for formalin-killed cells and between second and third bleeds for heat-killed cells reaching an absorbance of 2.448 and 2.453, respectively, both at 1:4000 dilutions.

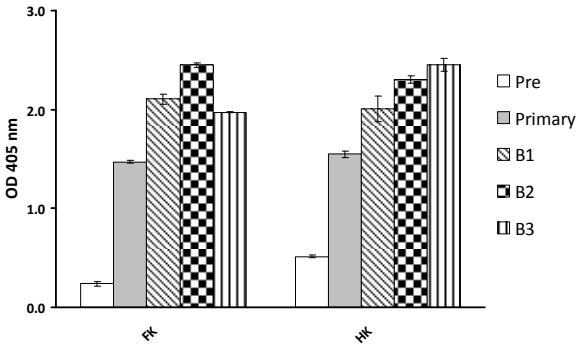

Fig. 1: Comparison of IgG titer between pre-immunized and booster serum for heat-killed (HK) and formalin-killed (FK) cells. The data were represented as mean ± SD (n=4).

The estimation of optimum immunoreagent concentrations forms the first step in the development of any immunochemical method to achieve maximum assay sensitivity and economic amount of PAb to be used. The constants of the calibration curve was then determined from suitable non-linear regression model. The concentration of the immobilized cells of the formalin-killed *Salmonella* Typhimurium ATCC 53648 was initially set at 10 x 109 CFU/mL. Optimum concentration of working concentration of PAb was found out to be 0.5 µg/mL of rabbit anti-IgY antibody-horseradish peroxidase conjugate. This concentration gave reasonably good detection and signal (Fig. 2).

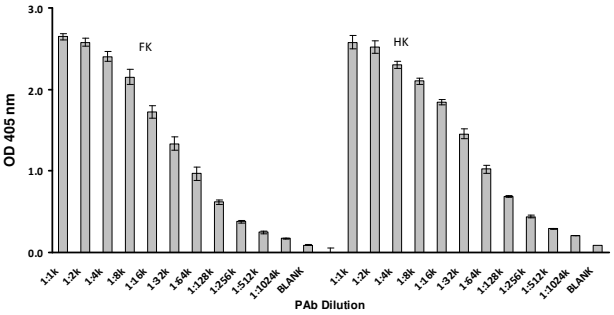

Fig. 2: Effect of serial dilutions of PAb raised against formalin-killed (FK) and heat-killed (HK) *S.* Typhimurium on assay activity measured after 30 minutes. Plate was coated with 100 µL/well of antigen with a concentration of $1x10^9$ cells/mL. The concentration of stock solution of PAb was 5 mg/ml and 100 µL of diluted PAb was used for each well. The data were represented as mean ± SD (n=4).

Determination of limits of detection

Under these conditions, the parameters of the typical calibration curve for formalin-killed were minimum absorbance of 0.101± 0.011, maximum absorbance of 2.804± 0.020, slope parameter was 1.197, while c, the concentration that results in 50% response was 4.22 x 106 CFU/ml (Fig. 3). The LOD and LOQ were 19,300 and 52,700 CFU/mL, respectively. The parameters of the typical calibration curve for heat-killed were minimum absorbance of 0.115± 0.013, maximum absorbance of 2.810± 0.021, slope parameter was 1.441, while c, the concentration that results in 50% response was 2.15 x 106 CFU/ml (Fig. 4). The LOD and LOQ were 47,100 and 123,000 CFU/mL, respectively. The linear region of the sigmoid calibration curve covers the concentration range of *Salmonella* Typhimurium ATCC 53648 for FK and HK was 500,000–100,000,000 and 500,000-10,000,000 CFU/mL, respectively. The shape of the curve represents the quality of antibodies.

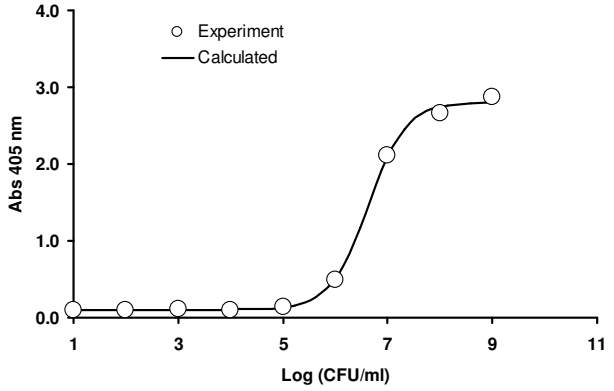

Fig. 3: Sensitivity of polyclonal antibody prepared through formalin-killed to *Salmonella* Typhimurium CFU/ml measured using indirect ELISA. The data were represented as mean ± SD (n=4).

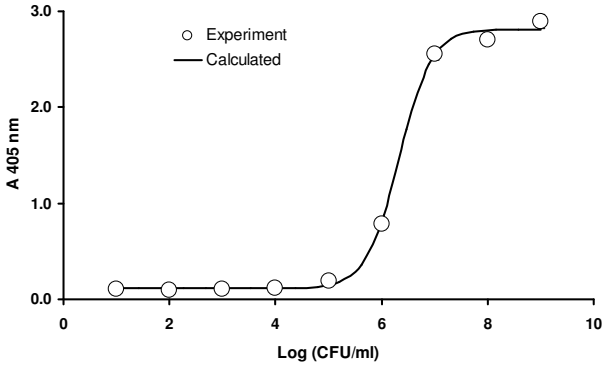

Fig. 4: Sensitivity of polyclonal antibody prepared through heat-killed to *Salmonella* Typhimurium CFU/ml measured using indirect ELISA. The data were represented as mean ± SD (n=4).

Specificity test

The specificities of the developed ELISA for both HK and FK were evaluated using a variety of foodborne pathogens including *E. coli* O157:H7, C. jejuni and *L. monocytogenes*. The results revealed that ELISA was sensitive towards both FK and HK *S.* Typhimurium with strong reactivity to HL E. coli while FK E. coli showed significantly ($p<0.05$) low cross reactivity. Low cross reactivity was also observed for both FK and HK cells of *C. jejuni* with FK cells exhibiting significantly ($p<0.05$) lower cross reactivity than HK. Both H and FK cells of *L. monocytogenes* showed very low cross reactivity (Fig. 5).

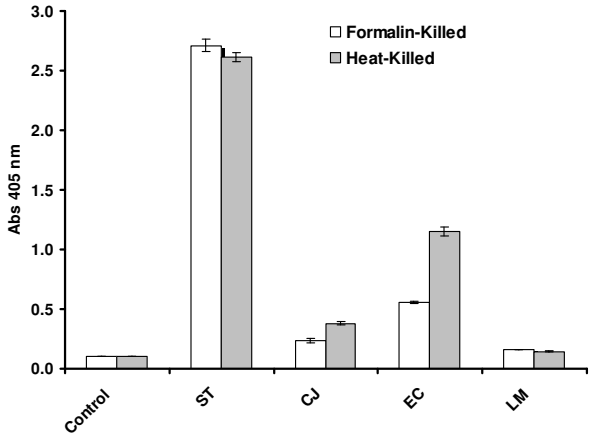

Fig. 5: Cross-reactivity of rabbit anti-*S.* Typhimurium IgG formalin-killed (A) and heat-killed (B) to *Salmonella* Typhi (ST), *Campylobacter jejuni* (CJ), *E. coli* (EC) and *Listeria monocytogenes* (LM). The data were represented as mean ± SD (n=4).

DISCUSSION

The early 1970s saw the development of the first ELISA [11]. The ELISA specifically developed to detect Salmonella was first reported in 1977 [12]. Numerous polyclonal- and monoclonal-based ELISA have been developed but the rise of newer mutants means that newer ELISA for Salmonella detection needs to be developed to increase the repertoire of arsenal important for detecting this pathogen that may escape detection by existing commercial ELISA-based assays specifically and other kinds of assays generally. Hence, in this work, we attempted to develop an IC-ELISA using PAb raised from heat- and formalin-killed strain of *S.* typhimurium not reported in the literature before.

Several other methods have been developed using either polyclonal or monoclonal antibodies to detect Salmonella serotypes [6,14]. However, high prevalence of Salmonella species frequently reported from variety of food and feed samples has encountered the need for more reliable detection methods. Although some ELISA methods have shown desirable limit to detect the Salmonella species in varied food and feed samples [15], there is still need to develop more ELISA-based methods for the real and widespread detection of *S.* Typhimurium in food and various samples [16].

The results for the antibody titer seen are typical of polyclonal antibody production from rabbit in a majority of works on *Salmonella* spp. detection. Antibodies level in rabbit during the immunization schedule allows a gradual increase in the apparent binding activity with boosting injections over the period tested. During this process serum maturation occurs revealing the typical pattern of primary and secondary response, during which specific antibodies appear after repeated immunizations.

The detection limits seen in this work are comparable to various formats of ELISA for Salmonella spp. such as Kumar et al. [17], Bautista et al. [18] and Bang et al. [19] where the detection limits lie between 10^4 and 10^6. Currently the most sensitive method for *Salmonella* spp. detection is electrochemical enzyme-linked immunosorbent assay (ELISA) coupled with flow injection analysis (ELISA-FIA) and a PCR-based method allowing detection limits down to 1 Salmonellae per 25 g sample [20].

Based on the minimum absorbance of 0.101 for FK and 0.116 for HK, the cutoff points to determine positive reaction were 0.200 and 0.230, respectively. A lower cutoff point of 0.135 was suggested by [17], based on the OD values they obtained in the negative control wells of between 0.060 and 0.075.

Overall, the result indicate that antibody raised to FK cells showed approximately twice the sensitivity and approximately ten times the linear range compared to antibody raised to HK cells. This is also observed by an indirect ELISA for the detection of *L. monocytogenes*. Their results showed between 2 and 10 times better sensitivity when using formalin-killed compared to heat-killed. On the other hand, another study showed that heat-killed cells gave better signal compared to formalin-killed *Listeria* spp. [21]. Cross reactivity is a major problem in polyclonal antibody ELISA system. Although some cross reactivity was seen, the results obtained in this work indicate that PAb produced from FK cells exhibited lower cross reactivity than HK and should be used in future studies.

In conclusion, highest antibody production was second bleed for formalin-killed cells and between second and third bleeds for heat-killed cells. The LOD and LOQ for formalin-killed were approximately half of that of heat-killed cells indicating better sensitivity of formalin-killed cells. In addition, antibody raised to FK cells showed approximately twice the sensitivity and approximately ten times the linear range compared to antibody

raised to HK cells. Moreover, the cross reactivity results indicate that PAb produced from FK cells exhibited lower cross reactivity than HK. It is hope that with the combination of clinical, molecular diagnostic and bacteriological methods, the use of IC-ELISA using PAb raised from HK and FK cells in would be able to improve diagnostic assay for the detection of *S.* Typhimurium in a variety of food and animal feed samples. Further studies using other commercial and conventional detection methods would be used to evaluate the efficacy of the developed method in detecting Salmonella in actual food, agricultural and even environmental samples.

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